Single Nucleotide Polymorphism in BCRP and Effects on Flavopiridol Transport

Linda Tran

Department of Pharmaceutics, Undergraduate Research Project, Spring 2008
The Ohio State University, Columbus, Ohio

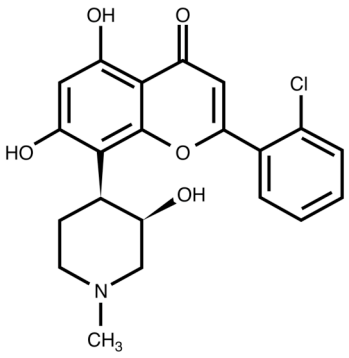


Figure 1: Flavopiridol

Abstract

Flavopiridol is a promising therapeutic agent currently under phase I and phase II clinical investigation for the treatment of Chronic Lymphocytic Leukemia (CLL) and other hematologic and solid tumor malignancies. Flavopiridol is a potent inhibitor of CDks (cyclin-dependent kinases), and its cytotoxic activity is associated with the arrest of cells in G_1 or G_2 phases of the cell cycle.[1] Breast cancer resistance protein (BCRP) is a 70 kDA transmembrane transporter involved in multidrug resistance.[2] BCRP exports flavopiridol from cells[3] resulting in decreased intracellular drug accumulation and resistance to its cytotoxic effects. A single nucleotide polymorphism in BCRP is known to change amino acid 141 from glutamine to lysine. To determine the effects on flavopiridol transport caused by this mutation, HEK-293 (human embryonic kidney) cells both stably and transiently transfected with wild-type and mutant BCRP were evaluated in flavopiridol cytotoxicity and transport assays. While early differences were observed in cytotoxicity assays with cells stably transfected with empty vector and BCRP, significant differences have not been observed with transiently transfected cells. In addition, no differences between the wild type and Q141K mutant were observed in both cytotoxicity assays comparing IC_{50} values and transport assays measuring the amount of intracellular flavopiridol. This report summarizes early efforts to develop the methods for evaluating the effects of the Q141K mutant. Further work to clarify the impact of this mutation on BCRP transport of flavopiridol is ongoing.

Introduction

CLL is the most common form of leukemia in western countries.[4] The disease is characterized by the clonal proliferation and accumulation of neoplastic B lymphocytes in the blood, bone marrow, lymph nodes, and spleen.[5] Approximately 15,500 new cases were diagnosed in 2007,[6] and 4,500 men and women will die from the disease each year[7]. With an average age of incidence at 62

years, CLL is an adult disease and only 10-15% of cases are people below 50 years of age.[4] At present, there is no curative therapy for CLL.[5]

Flavopirdol is a therapeutic agent currently in clinical trials for the treatment of hematological malignancies such as CLL. Recently, flavopiridol was shown to be active in CLL patients with a response rate of approximately 50%.[8] Flavopiridol is a potent inhibitor of CDks (cyclin-dependent kinases) which are involved in the regulation of the cell cycle. CDks are attractive targets for anti-cancer treatment because their interruption causes cell cycle arrest in either the G_1 or G_2 phase thus preventing mitosis and eventually leading to cell death.[1] Flavopiridol induces cell death by down-regulation of the anti-apoptotic proteins Mcl-1 and X-linked inactivator of apoptosis (XIAP)[9,10,11] through a p53-independent pathway[12,13]. Interestingly, CLL cells are non-dividing, but they maintain a high susceptibility to flavopiridol-induced cell death.

Flavopiridol is administered intravenously, and elimination occurs mainly by metabolism and biliary excretion. Metabolism takes place primarily through glucuronidation by the uridine diphosphate-glucuronosyltransferase (UGT) isoforms, UGT1A1 and UGT1A9. Glucuronide metabolites and parent drug are believed to be excreted into bile via the transporters BCRP and MRP2, ATP-binding cassette transporters with diverse substrate specificity.[14,15,16,17] Figure 2 displays a schematic of the hepatic/biliary elimination pathway, including transporters responsible for uptake and efflux.

BCRP is a 70 kDA transmembrane transporter that facilitates multidrug resistance.[2] BCRP exports flavopiridol from cells[3] resulting in decreased intracellular accumulation thereby providing a drug resistance mechanism. A single nucleotide polymorphism in BCRP is known to change amino acid 141 from glutamine to lysine. As a potential contributor of differential response to flavopiridol, genetic differences in BCRP may result in altered flavopiridol disposition and patient response. To determine the effects on flavopiridol transport caused by this mutation, HEK-293 (human embryonic kidney) cells both stably and transiently transfected were exposed to flavopiridol and analyzed using cytotoxicity and

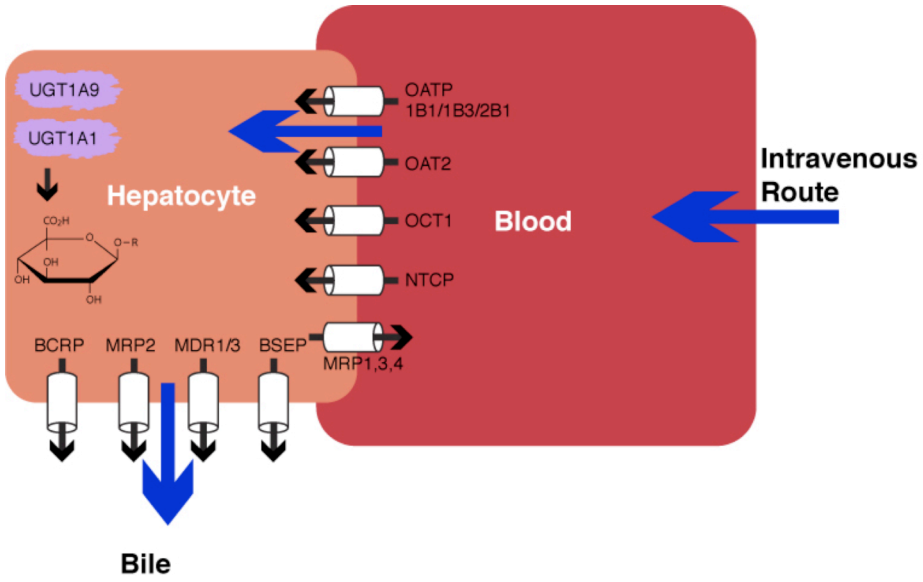


Figure 2: Flavopiridol Clearance and the Role of Transporters

transport/uptake assays.

Herein, efforts to evaluate the effects of the Q141K mutation on BCRP-mediated transport and resistance to the cytotoxic effects of flavopiridol are described.

Materials and Methods

Compounds

Flavopiridol (NSC 649890, HMR 1275, Alvocidib, MW 401.84) was obtained from the NCI as a hydrochloride salt with MW 438.29. The internal standard (IS), genistein (MW 270.24) was obtained from Sigma (St. Louis, MO), and SN-38 was purchased from LKT Laboratories (St. Paul, MN). All other chemicals were obtained from ThermoFisher Scientific (St. Louis, MO) unless otherwise noted.

Cell Culture

HEK-293 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and ~1% L-glutamine at 37°C with 5% CO_2. To maintain expression in the cells stably transfected with either BCRP or pcDNA3 empty vector, G418 was added to the medium at a concentration of 1mg/ml. Cells stably transfected with BCRP and pcDNA3 and BCRP and Q141K vectors were donated for this project by Dr. Duxin Sun.

Plasmid DNA Transfections

The transfection of empty pcDNA3, wild-type and Q141K mutant plasmids into HEK-293 cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) transfection reagent. One day prior to transfections, cells were seeded into a T-25 flask so that they would be ~90% confluent the following day. The DNA plasmids were diluted to 8,000 ng/flask in Opti-MEM I Reduced Serum Medium (Invitrogen), and Lipofectamine 2000 was diluted according to manufacturer's instructions. The diluted DNA and diluted Lipofectamine 2000 were combined, incubated for 20 minutes at room temperature then added to the T-25 flask containing cells and medium. The flasks were incubated at 37°C with 5% CO_2 for 24 hours prior to use.

Western Blot Analysis

Protein for the Western Blot analysis was isolated using cell lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1mM Na_2EDTA, and 1% Triton X-100). The protein was diluted with 2X loading buffer so that 30 ug of protein was loaded per well into a 10% SDS-PAGE gel. The gel was subjected to electrophoresis at 120 V for 90 minutes at room temperature. The gel was then electrotransferred onto a Trans-Blot® (Bio-Rad, Hercules, CA) pure nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBST and subsequently washed with TBST. Anti-BCRP mAb, clone BXP-21 (mouse IgG) was added for overnight incubation at 4°C with shaking. The membrane was washed again with TBST and then incubated with 2° mouse antibody (Ab) for 1 hour at room temperature on a shaker. The membrane had a final wash with TBST followed by detection with either the chemiluminescence (Amersham, Buckinghamshire, UK) or chromogenic (Vector Laboratories, Burlingame, CA) method.

Real-Time PCR

Extraction of mRNA was obtained using Trizol (Invitrogen) reagent. Cells were seeded in 24-well plates at a density of 1.0×10^5 cells per well in complete RPMI 1640 medium and allowed to incubate for 24 hours at 37°C with 5% CO_2. The media from each well was removed and replaced with 200 uL of Trizol reagent. The cell lysate was passed several times through a pipette tip with scrapping and transferred to a 1.5 mL centrifuge tube. The tube was centrifuged at 12,000xg for 10 minutes at 6°C and the resulting cleared homogenate solution was transferred to a new 1.5 mL centrifuge tube leaving behind the insoluble material. Subsequently, phase separation was achieved using 40 uL of chloroform per tube and the upper aqueous phase was transferred to a fresh 1.5 mL centrifuge tube. The RNA was precipitated by adding 100 uL of isopropyl alcohol to each tube. The tube was centrifuged at 8,000xg for 10 minutes at 6°C followed by removal of the supernatant, and washing of the RNA pellet with 200 uL of 75% ethanol. The tube was allowed to

air-dry for 10 minutes and the RNA was redissolved with 10 uL of Rnase-free water. Real-time, one-step RT-PCR for mRNA of BCRP was performed using the Power SYBR Green RNA-to-C_T™ 1-Step Kit (Applied Biosystems, Foster City, CA) protocol. Analysis of the data obtained from the experiment was performed using the method described by Livak KJ and Schmittgen TD[18]. RT-PCR was performed in the CCC Nucleic Acids Shared Resource.

Cytotoxicity Assays

Cytotoxicity assays were performed following the sulforhodamine B (SRB) assay procedure using the known BCRP substrate, SN-38, as a positive cytotoxic control agent. Cells were seeded in 96-well plates at a density of 20,000 cells per well for the stably transfected cells, and 40,000 cells per well for the transiently transfected cells. The cells were allowed to attach for 24 hours at 37°C with 5% CO_2. Either flavopiridol or SN-38 dosing solutions were prepared in RPMI 1640 medium without serum and were added to the cells at various concentrations (30-10,000 nM for flavopiridol and 1-1,000 nM for SN38), followed by incubation for 96 hours at 37°C with 5% CO_2. Subsequently, the cells were fixed in 4°C 10% trichloroacetic acid, then stained in 0.4% sulforhodamine B dissolved in 1% acetic acid. After 20 minutes at room temperature, the plates were washed with 1% acetic acid in H_2O and allowed to dry overnight in a fume hood. The bound dye was solubilized with 10mM Tris base and cell density was determined by measuring the absorbance at 540 nm. To investigate the Q141K mutant, PC-HEK (HEK-293 cells stably transfected with an empty vector) cells were established as the control. The flavopiridol IC_{50} value of PC-HEK was compared with those of R2-HEK (HEK-293 cells stably transfected with wild-type BCRP) cells, BCRP (HEK-293 cells transiently transfected with wild-type BCRP) cells, and Q141K (HEK-293 cells transiently transfected with mutant BCRP) cells.

Transport Assays

Stably transfected and transiently transfected cells were seeded in 24-well plates at a density of 1.0 x 10^5 cells per well in complete RPMI 1640 medium and allowed to incubate for 24 hours at 37°C with 5% CO_2. Dosing solutions were prepared in RPMI 1640 medium without serum and L-glutamine at a concentration of 1 uM for flavopiridol and 10 uM for SN-38. The media from each well was removed and replaced with dosing solution for 10 minutes. The dosing solution was removed and the wells were washed with 4°C versene. To each well, 50 uL of Trypsin-EDTA was added then plates were incubated for 10 minutes at 37°C. Afterwards, 300 uL of versene was added to each well, and the cells were resuspended. For each well, 150 uL was reserved to conduct the BCA Protein assay, and 200 uL was transferred to a 1.5 mL centrifuge tube. Added to each 1.5 mL centrifuge tube were 1 mL of 4°C ACN and 200 nM of Genistein, the internal standard. The tubes were vortexed and centrifuged at 16,000xg for 10 minutes. From each tube, 1 mL was removed and placed in a new 1.5 mL centrifuge tube. The tubes were dried in a speedvac. For each tube containing the dried sample, 150 uL of 95% H_2O, 5% ACN, 0.1% acetic acid was added, vortexed, and centrifuged at 16,000xg for 10 minutes. From each tube, 80 uL was removed and placed in an autosampler vial to be analyzed using LC/MS with a validated assay described by Phelps MA and Rozewski DM[19]. Once again SN-38 was used as the positive control. The data generated was compiled to determine if the Q141K mutant caused a change in the amount of intracellular flavopiridol.

Results

Real-Time PCR

RT-PCR measuring RNA levels confirmed expression of ABCG2, the gene that codes for the BCRP transporter. From the data obtained, Table1 shows that there is no difference seen between the PC-HEK control cells and the HEK-293 cells that do not contain the gene as expected. However, a significant difference of $\Delta\Delta C_T$ is seen in gene expression between the PC-HEK control cells and the transiently transfected BCRP and Q141K cells, respectively (n=6). This suggests that the DNA plasmid vectors have been successfully transfected into the HEK-293 cells, and gene transcription is occurring.

Table 1: Relative Expression of ABCG2 to PC-HEK

Cell / Transfection	Result 1 $\Delta\Delta C_T$	Result 2 $\Delta\Delta C_T$	Average Result $\Delta\Delta C_T$
PC	0.00	0.00	0.00
HEK	-0.44	0.21	-0.11
BCRP	11.93	12.23	12.08
Q141K	13.27	9.36	11.31

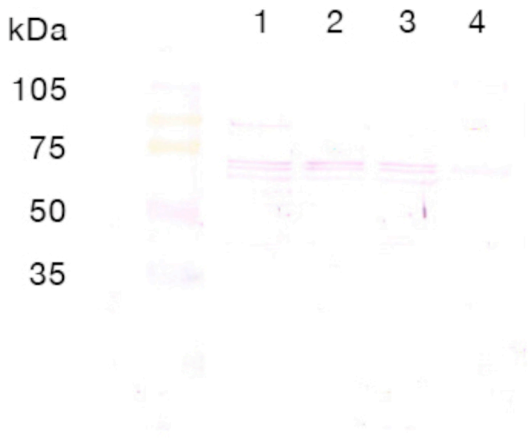
The results in $\Delta\Delta C_T$ for HEK, BCRP, and Q141K indicate the relative change in gene expression of the ABCG2 gene to the reference group, PC, which does not contain the gene.

Western Blot Analysis

Western Blot analysis was used to determine level of protein expression. Results from this analysis are inconclusive since equivalent signals are observed in BCRP transfected and control (empty vector transfected) cells. There is evidence that a protein with MW ~70 kDA is present, but that protein is also observed in control samples. This can be seen in Figure 3 with the chromogenic detection method where a band is seen at ~70 kDA for the empty vector control, wild-type BCRP, and mutant Q141K transfections. From these results, the protein detected is likely not BCRP but rather a random protein recognized by our antibody. Further work is required to develop the Western conditions.

Figure 3: Western Blot Analysis Using Different Detection Methods

Chromogenic Detection



1. PC w. pcDNA3 transfection
2. PC w. BCRP transfection
3. PC w. Q141K transfection
4. R2

Chemiluminescence Detection

1. PC w. BCRP transfection
2. PC w. Q141K transfection

Cytotoxicity Assays

Cytotoxicity assays were performed to evaluate the effects caused from the Q141K mutation on both flavopiridol and SN-38 cytotoxicity when compared to the wild-type BCRP. Table 2 shows that when cells were transfected with either the wild-type or mutant BCRP transporter, the IC_{50} values showed a slight increase suggesting that the transporter is eliminating drug from the cells. The results were consistent in that both transfections caused an increase in the amount of drug it takes to kill the cells, but there is no difference observed between the wild-type and mutant. Figure 4A shows a representative dose response curve for HEK cells treated with flavopiridol. Figure 4B shows a representative dose response curve from BCRP transiently transfected cells treated with flavopiridol. In figure 4B, a baseline level of cells remains even at higher doses of drug, indicating a higher tolerance for flavopiridol. This suggests that there could be two populations of cells in the studies with transiently transfected cells. One population is of the cells that are not expressing BCRP and are dying at low drug concentrations. The other population is that of cells expressing BCRP which have an apparently higher tolerance for drug.

Table 2: Flavopiridol Resistance in Transiently Transfected Cell Lines

EXP. #	Cell / Transfection	IC_{50} Flavopiridol	IC_{50} SN-38
1	PC	1.0	1.0
1	R2	1.3	15
2	PC	1.0	-
2	BCRP	1.2	-
3	PC	1.0	1.0
3	BCRP	1.4	3.8
4	PC	1.0	1.0
4	BCRP	1.1	1.3
4	Q141K	1.1	1.0
5	PC	1.0	1.0
5	BCRP	1.3	1.3
5	Q141K	1.3	1.0

Numbers represent ratio of IC_{50} of BCRP-transfected vs. control

Figure 4A: Dose Response Curve of Stably Transfected PC-HEK Cells Treated with Flavopiridol (nM)

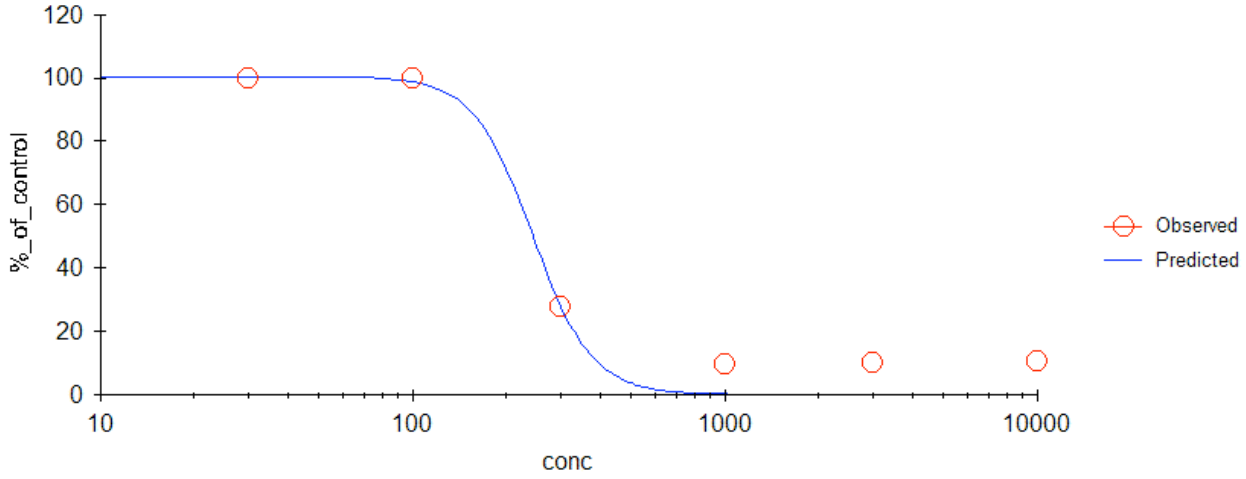
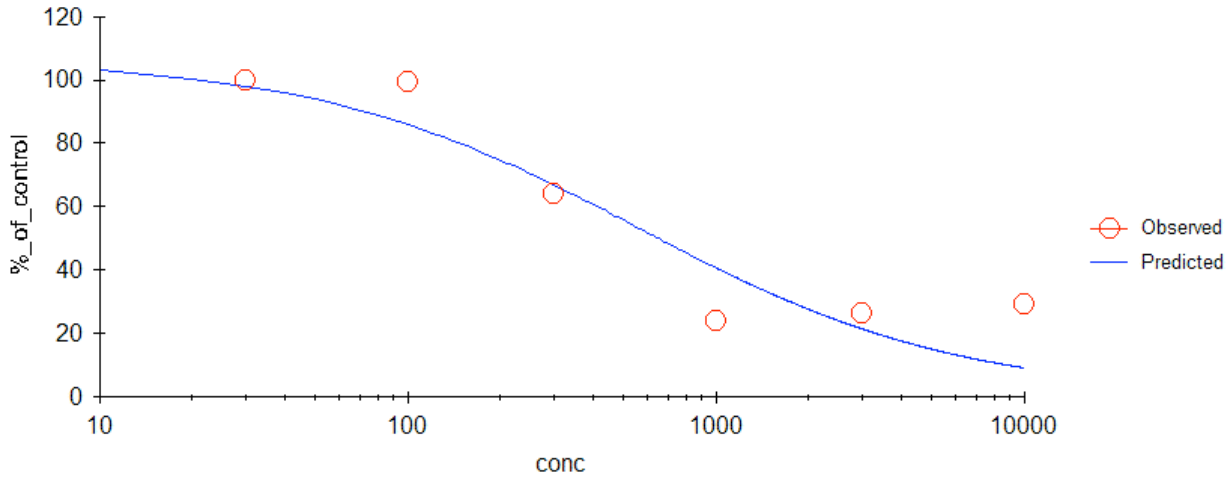


Figure 4B: Dose Response Curve of Transiently Transfected BCRP Cells Treated with Flavopiridol (nM)



Transport Assays

The Transport assays provided data which agrees with the trend seen in cytotoxicity assays. Based on the transport velocity, BCRP acts to eliminate drug from cells, but there is no difference seen in the rate of drug elimination between the wild-type BCRP and mutant Q141K transporter. Figure 5 indicates that the intracellular flavopiridol transport velocity in *p*mol/min are approximately the same for the wild-type BCRP and mutant Q141K transporter. In Figure 6 using SN-38 as the positive control, the trend in relative transport velocity rate is again illustrated, although to a lesser degree. In both figures, wild-type BCRP and mutant Q141K are seen behaving very similarly in the rate of drug elimination. However, none of the trends observed are statistically significant.

Figure 5: Flavopiridol Upatke in Transfected HEK-293 Cells (1uM, 10 min., n=5). Numbers represent P values compared to PC-HEK

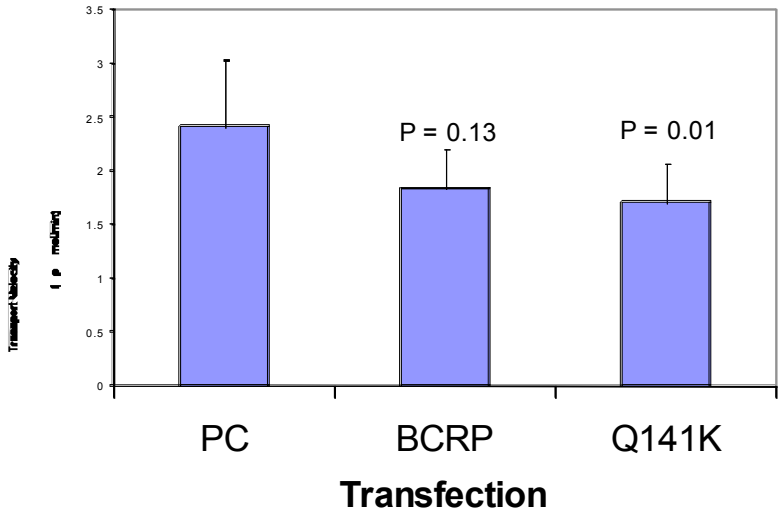
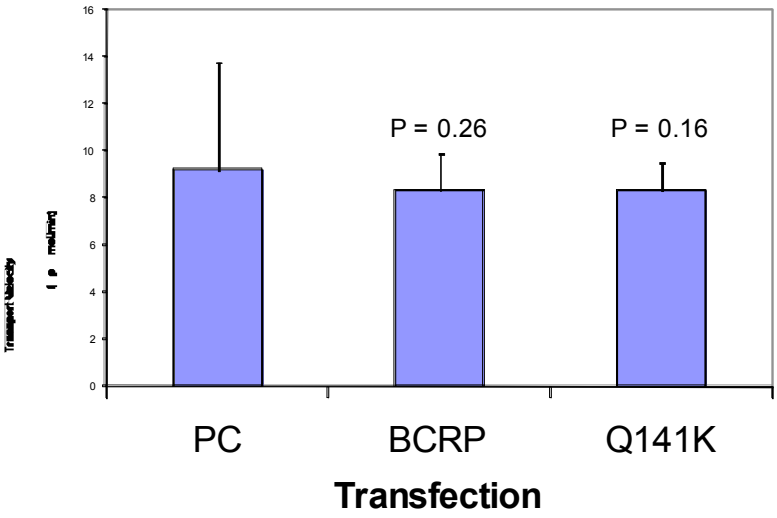


Figure 6: SN-38 Uptake in Transfected HEK-293 Cells (10uM, 10 min., n=5). Numbers represent P values compared to PC-HEK



Discussion

RT-PCR experiments were successful in revealing transcription of the ABCG2 gene, but it was not able to confirm that BCRP was being translated. To detect the presence of BCRP, the use of Western blots was attempted. These experiments were not successful due to apparently low recovery of the membrane transport proteins from the cell lysates. Additional experiments were planned to use a stronger cell lysis buffer (SDS and urea) for better solubilization of membrane proteins, and these experiments are in progress.

Both cytotoxicity assays and transport assays indicate small differences in mean uptake IC_{50} and velocity, respectively, between cells that contain BCRP and cells that do not. The difference seen however is not statistically significant, and indicates a need to improve the assays. This will be done with stably transfected cells with high levels of protein expression. Although some cells stably transfected with BCRP were donated for this project, these were not used at all times during the project due to the difficulty of recovering them from their frozen state in liquid nitrogen and the difficulty in maintaining healthy passages.

Even though the results in these studies indicate trends for reduced cytotoxicity and increased transport between BCRP or Q141K transfected vs. controls, the differences are small and statistically not significant. This is true for both SN-38 and flavopridol, which are both known substrates for BCRP. Additionally, no differences in cytotoxicity or transport between BCRP and the Q141K mutant are seen. Further work is ongoing to improve the results obtained to date. This work includes evaluation of different conditions for Western Blot analysis, and development of stably transfected cell lines overexpressing BCRP and Q141K mutant.

Further experiments using techniques that this project has helped to develop may yield stronger evidence to support the results of this project. As flavopiridol continues through clinical trials, information that is generated about the drug from this and other studies become important due to the potential direct impact on treatment outcome.

References

1. Takeo Nakanishi, Judith E. Karp, Ming Tan, L. Austin Doyle, Todd Peters, Weidong Yang, David Wei, and Douglas D. Ross, “Quantitative Analysis of Breast Cancer Resistance Protein and Cellular Resistance to Flavopiridol in Acute Leukemia Patients.” Clin. Cancer Res. 2003 9: 3320-3328.
2. Kamiya Biomedical Company, Product Data Sheet. http://www.kamiyabiomedical.com/
3. Doyle LA, Ross DD (2003). "Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2)." Oncogene 22 (47): 7340-58
4. World Health Organization, Chronic Lymphocytic Leukemia Treatment Guidelines. www.emro.who.int/ncd/publications/CLL.pdf
5. Rozman C, Montserrat E: Chronic lymphocytic leukemia. N Engl J Med 333 (16): 1052-7, 1995.
6. The Leukemia & Lymphoma Society, Disease Information. http://www.leukemia-lymphoma.org/all_page?item_id=7059
7. National Cancer Institute, SEER Cancer Statistics Review. http://seer.cancer.gov/statfacts/html/clyl.html
8. Byrd, J. C., T. S. Lin, et al. (2007). "Flavopiridol administered using a pharmacologically derived schedule is associated with marked clinical efficacy in refractory, genetically high-risk chronic lymphocytic leukemia." Blood **109**(2): 399-404.
9. Gojo I, Zhang B, Fenton RG: The cyclin-dependent kinase inhibitor flavopiridol induces apoptosis in multiple myeloma cells through transcriptional repression and down-regulation of Mcl-1. Clin Cancer Res 8:3527-38, 2002.
10. Kitada S, Zapata JM, Andreeff M, et al: Protein kinase inhibitors flavopiridol and 7-hydroxy-staurosporine down-regulate antiapoptosis proteins in B-cell chronic lymphocytic leukemia. Blood 96:393-7, 2000.
11. Wittmann S, Bali P, Donapaty S, et al: Flavopiridol down-regulates antiapoptotic proteins and sensitizes human breast cancer cells to epothilone B induced apoptosis. Cancer Res 63:93-9, 2003.
12. Parker BW, Kaur G, Nieves-Neira W, et al: Early induction of apoptosis in hematopoietic cell lines after exposure to flavopiridol. Blood 91:458-65, 1998.
13. Byrd JC, Shinn C, Waselenko JK, et al: Flavopiridol induces apoptosis in chronic lymphocytic leukemia cells via activation of caspase-3 without evidence of bcl-2 modulation or dependence on functional p53. Blood 92:3804-16, 1998.
14. Jager W, Gehring E, Hagenauer B, et al: The role of hepatic Mrp2 in the interaction of flavopiridol and bilirubin: impact on therapy. Int J Clin Pharmacol Ther 41:610-1, 2003.
15. Nakanishi T, Karp JE, Tan M, et al: Quantitative analysis of breast cancer resistance protein and cellular resistance to flavopiridol in acute leukemia patients. Clin Cancer Res 9:3320-8, 2003.
16. Robey RW, Medina-Perez WY, Nishiyama K, et al: Overexpression of the ATP-binding cassette half-transporter, ABCG2 (Mxr/BCrp/ABCP1), in flavopiridol-resistant human breast cancer cells. Clin Cancer Res 7:145-52, 2001.
17. Jager W, Gehring E, Hagenauer B, et al: Biliary excretion of flavopiridol and its glucuronides in the isolated perfused rat liver: role of multidrug resistance protein 2 (Mrp2). Life Sci 73:2841-54, 2003.
18. Livak KJ and Schmittgen TD, “Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method.” Methods 2001 25: 402-408.
19. Phelps, M. A., D. M. Rozewski, et al. (2008). “Development and validation of a sensitive chromatography/mass spectrometry method for quantitation of flavopiridol in plasma enables accurate estimation of pharmacokinetic parameters with a clinically active dosing schedule.” J. Chrom. B In press.

Acknowledgement
The work in this project was completed under the careful guidance of Dr. Mitch Phelps of The Ohio State University.